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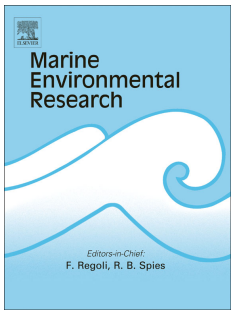

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Genetic polymorphism and its potential relation to environmental stress in five populations of the European flounder *Platichthys flesus,* along the French Atlantic Coast

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Abstract

In this study, new DNA markers were explored for the flounder *Platichthys flesus*. cDNA and genomic sequences of the genes encoding the glyceraldehyde-3-phosphate-deshydrogenase (GAPDH), the cytosolic creatine kinase (CK), the prostaglandin D synthase (PGDS) and the betain homocystein methyltransferase (BHMT) were characterized. The tumour suppressor p53 gene structure was already described. A PCR-SSCP (*Single Strand Conformation Polymorphism*) analysis was finally conducted to study the genetic polymorphism of different populations of flounders collected along the French Atlantic coast. Four highly contaminated French estuaries (Seine, Vilaine, Loire and Gironde) were sampled and compared to a reference estuary (Ster) to explore possible selective effect of the environment on specific allelic frequencies. Our results showed that two loci p53and PGDS, could be potential markers of chemical stress: p53A allele frequency increased in contaminated systems compared to the reference system. In the Vilaine estuary, PGDS polymorphism could be related to pesticide stress.

Keywords: flounder; contamination; gene; polymorphism; PCR-SSCP; selection

1. Introduction

Coastal ecosystems have been subjected for several decades to increased anthropogenic pollution (hydrocarbons, pesticides…) and to other environmental stressors (hypoxia, temperature increase, eutrophication …). Some of these stressors are known to induce modifications of the genetic structure of populations living in these environments (Gillespie and Guttman, 1993; Moraga et al., 2002; Tanguy et al., 2002), retaining the fittest genotypes in the perturbated systems and thus conducting to changes in allelic frequencies (Ma et al., 2000; Bickham et al., 2000). Moreover, modifications of the genetic variability within and between populations may reduce their adaptability to new environments. Allelic variation at any loci that contribute to a modification of the phenotype could be acted upon by selection (Carvalho, 1993). Therefore, specific genotypes may be selected in natural populations because they contribute to the resistance to the toxic effects of pollutants; the relative proportion of these "resistant" genotypes may increase in chronically contaminated populations (Heithaus and Laushman, 1997; Larno et al., 2001).

Allozyme markers have been extensively studied on aquatic organisms, particularly to evaluate the impact of chemical stress on the genetic structure of fish populations living in heavily contaminated rivers (Gillespie and Guttman, 1993, 1999; Benton et al., 1994; Foré et al., 1995; Heithaus and Laushman, 1997). But few studies focused directly on the genetic polymorphism of specific candidate genes, at the DNA level. Thus, the aim of this study was to characterize new nuclear genetic markers in the European flounder *Platichthys flesus*. We analyzed the variability of five genes: the glyceraldehyde-3-phosphate deshydrogenase (GAPDH), the cytosolic creatine kinase (CK), the prostaglandin D synthase (PGDS), the betain homocystein methyltransferase (BHMT) and the p53 genes. Four of them (GAPDH, CK, PGDS and BHMT) were partially isolated in a previous work performed on flounders exposed to different pesticides, and differentially regulated during the exposition (Marchand et al., 2006). The tumour suppressor gene p53 was characterized previously (Cachot et al., 1998, 2000)

The glyceraldehyde-3-phosphate dehydrogenase (GAPDH) enzyme is a classical glycolytic protein playing an essential role in the carbohydrate metabolism. Firstly, the GAPDH mRNA level has been commonly used as an invariant internal standard for various gene expression assays, considering that this gene would be constitutively expressed. However from the last decade, it was demonstrated that GAPDH could be a multifunctional

modulator implicated in diverse cellular pathways, especially those involved in induced apoptosis and neurodegenerative disorders (Chuang et al., 2005; Sirover, 2005).

Creatine kinase (CK) enzymes, specifically located in sites of energy demand or production, are linked to a phosphocreatine/creatine circuit found in particular cells. Two CK isoforms may be found in vertebrate skeletal muscle: a cytosolic isoform (designated MM-CK) and a mitochondrial isoform (designated Mt-CK) (Wallimann et al., 1992). Phosphocreatine production, catalyzed by Mt-CK, is favoured at the mitochondrial membrane level. The reverse reaction is favoured at sites of ATP utilization and is catalyzed by MM-CK (Hochachka et al., 1983; Wallimann et al., 1992). Transgenic mice whose skeletal muscles are deficient in MM-CK are incapable of burst activity (van Deursen et al., 1993).

The betaine homocysteine methyltransferase (BHMT) enzyme catalyzes a key reaction at the convergence of the folate and the methionine cycles. This enzyme plays an important functional role in homocysteine remethylation (Li et al., 2008). Homocysteine is a highly reactive compound; highest concentrations inducing oxidative damage (Refsum et al., 1998). As many different methyltransferases (Hodgson and Levi, 1992), BHMT may possibly act as a phase II metabolizing enzyme. Despite intense interest in elevated circulating homocysteine levels as a possible risk factor for human cardiovascular disease, osteoporosis, dementia, and complications of pregnancy (Hermann, 2006), surprisingly little is known with regard to common genetic variation, even in humans.

The lipocalin-type prostaglandin D synthase (L-PGDS) is a bifunctional protein possessing both the ability to synthesize prostaglandins and to serve as a carrier protein for lipophilic molecules (Fujimori et al., 2006). L-PGDS has been extensively studied in mammalian species, whereas little is known about non-mammalian forms. L-PGDS concentrations are useful for the diagnosis of several profound disorders (neurological, cardiovascular, and renal) and multiple sclerosis and cancers in humans (Su et al., 2001; Hirai et al., 2001; Chen et al., 2004). Moreover, Vogel (2000) showed that not only endogenous stimuli but also drugs and environmental chemicals can activate prostaglandin D synthase expression, especially in certain target tissues that possess low CYP monooxygenase activity. In particular, Hodgson and Levi (1991, 1992) showed that pesticides may be metabolized by prostaglandin synthetase.

To our knowledge very few studies have been published on the DNA polymorphism of the four previous genes and most concerned humans (Tso et al., 1985; Greenblatt et al., 1994; Li et al., 2008).

The last studied gene is the tumour suppressor p53 gene. The human homologue encodes a 53 kDa transcription factor which regulates cell cycle, apoptosis and DNA integrity in response to stress factors such as DNA damage, oncogene activation or hypoxia (see Pluquet and Hainaut, 2001). To date, this gene has been partially or completely sequenced in about eighteen fish species including the European flounder (Cachot et al., 1998). Although the p53 gene sequence has diverged in the course of vertebrate evolution (Cachot et al., 2000), some of the critical functions are conserved. Indeed, fish p53 acts as a transcription factor which inhibits cell growth (Langheinrich et al., 2002; Cachot et al., 2004) and promotes apoptosis in response to DNA-damage induction (Langheinrich et al., 2002; Berghmans et al., 2005). In contrast to human, fish p53 gene is infrequently mutated in sporadic and chemically-induced tumours (Cachot et al., 2000; Franklin et al., 2000; Sueiro et al., 2000).

In this study, the isolation and characterization of the full-length cDNA and genomic sequence that encodes *P. flesus* GAPDH, CK, BHMT and PGDS are described, the p53 gene sequence being already described by Cachot et al. (2000). A PCR-SSCP (*Single Strand Conformation Polymorphism*) study was finally conducted to study the genetic polymorphism of different populations of flounders collected along the French Atlantic coast and to explore possible selective effects of the environment on specific allelic frequencies.

2. Materials and methods

Biological model

The European flounder *Platichthys flesus (L.)* is a benthic flatfish commonly distributed in the East Atlantic Ocean. During its juvenile period, it lives in estuaries and brackish waters. This species has been used for several decades as a sentinel species for pollution monitoring in the North-East Atlantic (Vethaak et al., 2009).

DNA markers

cDNA characterization

The characterization of four *P. flesus* cDNA sequences was performed: the glyceraldehyde-3-phosphate deshydrogenase (GAPDH), the creatine kinase (CK), the prostaglandin D synthase (PGDS) and the betain homocystein methyltransferase (BHMT). The procedures for the generation of cDNA of the 5’ and 3’ untranslated regions (UTRs) for

these four sequences were carried out according to the commercial protocol for the rapid amplification of 3'/5' cDNA ends (5'/3' RACE Kit, Roche, Mannheim, Germany), using specific primers. Primers were designed based on the partial cDNA sequences of *P. flesus* GAPDH, CK, PGDS and BHMT previously identified (Marchand et al., 2006): Table 1. Total RNA was extracted from the hepatopancreas and the muscle of *P. flesus* according to the method based on extraction in guanidium isothiocyanate (Strohman et al., 1977). cDNA was synthesized from 20µg of RNA using the oligo dT race primer (Table 1), 2mM dNTPs, and M-MLV reverse transcriptase (Promega, Madison, WI, USA), and was then purified using the Wizard® DNA Clean-Up System (Promega).

200 ng of reverse-transcribed products were used for the PCR amplification of the 3'UTRs in a final volume of 25µl with 1x reaction buffer (Interchim), 1.5mM $MgCl_2$, 0.04mM dNTPs mixture, 1µM of the race primer and the specific reverse primer R_1 (Table 1), 0.25U of Taq Uptitherm DNA polymerase (Interchim) and submitted to the following program in a thermocycler (Applied Biosystems): 94°C for 2 min, followed by 35 cycles at 94°C for 30 s, Ta°C for 30 s and 72°C for 1 min and an additional step at 72°C for 10 min.

Amplification of the 5' UTRs were carried out according to the following procedure: 200 ng of reverse transcription product was treated with terminal deoxynucleotidyl transferase (Promega, Madison, WI, USA) and dATP to generate a polyA tail at the 5' end. A first PCR amplification was performed on this product with 1µM of the oligo dT race primer and the specific forward primer F_1 (Table 1) and submitted to the following program: 94°C for 2 min, followed by 15 cycles at 94°C for 15 s, Ta°C for 30 s with an increase of 0.2°C per cycle, 72°C for 2 min, then 25 cycles at 94°C for 15 s, Ta+3°C for 30 s, 72°C for 2 min, and a final step at 72°C for 10 min. A second PCR amplification was performed using 1µl of the amplification products, 1µM of the race primer and the specific forward primer 2 (Table 1), and submitted to the program cited above.

PCR products were analyzed on a 1X TAE (Tris-Acetate-EDTA)/1% agarose gel (130 Volts for 1 hour), visualized with UV light after staining with ethidium bromide, excised from agarose and purified using the QIAEX II Gel Extraction Kit (Qiagen) following the manufacturer's instructions. Purified PCR products were then ligated to the pGEM-T easy plasmid vector (Promega) and transfected into *Escherichia coli* DH5α strain. Recombinant bacteria were identified by blue/white screening and white colonies were grown in Luria-Bertani medium (with 100 $mg.L^{-1}$ ampicillin) from which the vector was extracted using an alkaline lysis plasmid preparation. Size screening was performed by PCR amplification using T7 and SP6 universal primers and selected clones were sequenced using a LiCOR IR^2

(Sciencetech, Lincoln, NE, USA) and the Thermo Sequenase Primer Cycle Sequencing Kit (GE Healthcare Europe, Freiburg, Germany). Sequences obtained were then subjected to a homology search through the BLASTX program available at the NCBI Genbank biocomputing site (http://www.ncbi.nlm.nih.gov/BLAST/) (Altschul et al., 1990). The deduced amino acid sequences were obtained using the translate software available at the ExPASy (Expert Protein Analysis System) server of the Swiss Institute of Bioinformatics (http://www.expasy.org/tools/). Their molecular weight and isoelectric point were calculated using the MWCALC software (Infobiogen, France). Entire cDNA sequences obtained were deposited in the NCBI Genbank biocomputing site.

Gene characterization

Genomic DNA was extracted from fin tissue. About 100mg of fin were placed in extraction buffer (0.3 M Tris, pH 8, 0.02 M ethylene diamine tetra-acetic acid [EDTA], 0.1M NaCl) together with sodium dodecyl sulphate (SDS) and proteinase K at a final concentration of 0.6% and 0.1 $mg.ml^{-1}$, respectively. The mixture was then incubated at 55°C until complete dissolution of tissue. NaCl was then added to a final concentration of 1.3 M. After homogeneisation, samples were centrifuged at 3000g at 20°C for 10 min. The supernatant was taken and two phenol/chloroform/isoamyl alcohol (25:24:1) extractions performed. DNA was precipitated with absolute ethanol, recovered by centrifugation 30min at 12000g at 4°C, rinsed with 70° ethanol, dried and dissolved in 1mL of TE buffer (10 mM Tris, pH8, 1 mM EDTA).

The different gene sequences were amplified using primers combinations based on the cDNA sequences identified previously (Table 2). Two hundred nanograms of genomic DNA, 1.5mM $MgCl_2$, 0.04mM dNTPs mixture, 1µM of each primer and 0.25U of Taq Uptitherm DNA polymerase were submitted to the following amplification: 94°C for 5 min, followed by 40 cycles at 94°C for 30 s, Ta°C for 40 s, 72°C for 1 min 30 s, and a final step at 72°C for 10 min. Cloning and sequencing were performed according to the procedures described previously and the gene sequences were deduced from the overlapping fragments sequenced and deposited in the NCBI Genbank biocomputing site.

PCR-SSCP (Single Strand Conformation Polymorphism) analysis

The SSCP analysis is one of the simplest, most reliable and most sensitive method for detection of mutations based on PCR (Sheffield et al., 1993) and this technique allows the detection of 99% of the mutations from fragments of 200 to 300 bp length (Orita et al., 1989).

For each gene, primers were designed to amplify each exon (except the too short exons) or two adjacent exons. We have optimized the technique to detect only single nucleotide polymorphisms (SNPs). Afterwards, a selection of SNPs was made, based on the polymorphism level and on the reproducibility of the band patterns. We have thus chosen: exon 3/4 for the GAPDH gene, exon 5/6 for the CK gene, exon 3 for the BHMT, exon 4/5 for the PGDS gene and exon 5 for the p53 gene (**Y08919**). Primers used for these amplifications are presented in Table 3. All PCR amplifications were performed in a volume of 25µL containing 1× Taq polymerase buffer, 2mM MgCl2, 40µM deoxynucleotides (dNTPs), 1µM of each primer, 0.5 units of Taq Uptitherm DNA polymerase and about 100 ng of total genomic DNA and submitted to the following program: 94°C for 5 min, 40 cycles of 30 s at 94°C, 40 s at Ta°C, and 1 min 30 s at 72°C, with a final elongation of 10 min at 72°C. The PCR products were then combined with 20µl of loading buffer (bromophenol blue, xylem cyanol, saccharose), heated for 5 min at 95°C, then rapidly chilled on ice to melt and retain single strand DNA. After loading on a neutral 8% polyacrylamide gel (37.5:1, acrylamide: bisacrylamide), the samples were electrophoresized at a constant voltage (120 V) in a 0.6× TBE buffer, for 16-20 h at either ambient temperature (PGDS and p53) or 4°C (GAPDH, CK and BHMT). After electrophoresis, the gels were stained with ethidium bromide and visualised under ultraviolet light. Single strand DNA bands from the PCR products visualized on the gel were gel-purified by diffusion into water by freezing at −20◦C and thawing. Double-strand DNAs were obtained from these recovered DNAs by PCR amplification using the same primers as for SSCP. PCR products were then purified, cloned and sequenced as described above. The SWISS Model 3.5 software available via the ExPASy server (Schwede et al., 2003) was used to determine the secondary structure of the different variants when non-synonymous changes occurred.

Studied sites and sample processing

Adult flounders were collected in four French polluted estuaries (Gironde, Loire, Seine and Vilaine) and one moderately contaminated estuary (Ster) considered as the ‘reference site’ (low domestic, agricultural and industrial effluents) (Marchand et al., 2003; Figure 1). The Gironde, Loire, Seine and Vilaine estuaries are subjected to strong anthropogenic influences and are chronically polluted by mixtures of chemicals. The Seine undergoes a diffuse contamination (complex mixture of organic chemicals) similar to the one reported in heavily polluted estuaries of North America (Munschy et al., 1997; Cachot et al., 2006). The chemical stress of the Loire estuary is rather similar but the level of organic

pollutant concentrations is however three times lower than the level found in the Seine estuary (Marchand et al., 2003). The Gironde estuary is mainly characterized by high levels of heavy metals such as zinc, cadmium and copper (RNO, 2001; Marchand et al., 2003) and water analyses carried out in the Vilaine estuary, between 1996 and 1999, displayed particularly high levels of pesticides (especially atrazine, diuron and isoproturon) (Forget, 1998; SAGE Vilaine, 2000) as a result of intensive agriculture. The Vilaine estuary (and the Loire to a lesser extent) is also known to experience hypoxic events (Menesguen et al., 2001).

A recent study underlined that chemical concentrations detected in flounder tissues allowed to consider the Ster estuary as a reference site, displaying a low level of contaminants (PCBs, PAH metabolite, metals) compared to other estuaries (Evrard et al., 2010). Furthermore, several studies confirmed that the average flounder growth rate was consistently higher in the Ster estuary compared to the polluted systems: Seine, Vilaine, Loire, Gironde (Laroche et al., 2002; Marchand et al., 2003, 2004; Evrard et al., 2010). As no North–South increase of the growth rate was observed over the contaminated estuaries, we suggest that globally the thermal regimes of these systems are not significantly different.

Forty flounders from each estuary were caught in winter (January and February 2003) by gillnets (Ster) and trawling operations (Gironde, Loire, Seine and Vilaine). The average sex ratio (males/females) and age (otolith observation) were estimated for the Ster, the Gironde, the Loire, the Seine and the Vilaine respectively: 0.29 and 2.7±0.3 years; 0.48 and 2.6±0.2 years; 0.14 and 2.7±0.2 years; 0.29 and 2.7±0.3 years; 0.58 and 1.2±0.2 years. Muscle and hepatopancreas were collected from each individual, flash-frozen in liquid nitrogen for RNA extraction (cDNA characterization), brought back to the laboratory and stored at -80°C until use. Fragments of fin were also preserved in alcohol for DNA extraction.

Genetic analysis

Statistical and population genetic analysis

The population genetic parameters (allelic frequencies for each locus, observed heterozygosity (Ho), expected heterozygosity (He) based on Hardy-Weinberg equilibrium) were calculated per population with the GeneClass2 software (Piry et al., 2004). Allelic frequencies were analysed with χ^2 conformity tests to detect potential heterogeneity between contaminated and uncontaminated populations. Differences in heterozygosity between the polluted and control sites were evaluated with a paired *t*-test using the locus as unit of replication (Leberg, 1992). Deviation from Hardy-Weinberg equilibrium was determined

within each population for each locus by computing the inbreeding coefficient Fis with the GENETIX 4.05 software (Belkhir et al., 2004). The significance of Fis was tested with the GENEPOP 3.2 software (Raymond and Rousset, 1995) using the Markov chain method (10 000 dememorisation steps, 1000 batches, 3000 iterations) to obtain unbiased estimates of the exact p-value (Guo and Thompson, 1992).

The genetic differentiation was assessed using Wright's F_{ST} (Wright, 1969) and exact tests of population differentiation (Raymond and Rousset, 1995). Single and multi-locus global F_{ST} was estimated using Weir and Cockerham's θ (Weir and Cockerham, 1984) with the GENETIX 4.05 software (Belkhir et al., 2004) and tested by permutated data sets (5000). Single and multi-locus global exact test of population differentiation was calculated with the GENEPOP 3.2 software (Raymond and Rousset, 1995) using a Markov chain method (10 000 dememorisation steps, 1000 batches, 3000 iterations). Furthermore, Fst by locus was assessed for each pair of locations. Control of the false discovery rate (FDR test) was carried out in multiple testing (Benjamini and Yekutieli, 2001).

3. Results

Molecular characterization

The accession numbers for the different cDNA and DNA sequences are presented in Table 4 as well as the length of the coding region (and the corresponding amino acids, molecular weight and isoelectric point), the length of the 5' and 3' UTR sequences and the size of the corresponding gene. The exon/intron structure for each gene is presented in Figure 2. All the introns of the different genes start and end with the consensus GT and AG splicing signals.

For the GAPDH cDNA, the typical eukaryotic GAPDH signature ASCTTNCL related to the substrate binding was found from the amino acid positions 148 to 155 (Sirover, 1999). Amino acids putatively related to the NAD^+ binding (D and E in amino acid positions 37 and 315) and the sites putatively related to the inorganic phosphate binding were also found (S, T, D and T in positions 149, 151, 196 and 209) in the GAPDH sequence of *Platichthys flesus* (Aoki et al., 2000; Liaud et al., 2000). For the cytosolic CK cDNA, lysine (K) charge clamp residues (Hornemann et al., 2000) were found in the CK sequence of *Platichthys flesus* in amino acid positions 105 and 116. These residues have been implicated in binding of mammalian MM-CK to the sarcomere M-line (Hornemann et al., 2000), allowing MM-CK to

be coupled directly to myosin ATPase activity (Wallimann and Eppenberger, 1985). The consensus sequence AATAAA was also present in the four sequences described in this study, respectively 20bp, 12bp, 16bp and 15 bp upstream from the polyadenylation site for GAPDH, CK, BHMT and PGDS.

Polymorphism analysis

Genetic variability within population

PCR-SSCP analysis performed at 5 loci in 189 flounders from four different estuaries allowed us to characterize: 5 alleles for GAPDH and PGDS, 4 alleles for CK and 2 alleles for BHMT and p53. Allelic frequencies and multi-locus heterozygosities are presented in Table 5. For GAPDH locus, a decrease of A allele frequency and an increase of B allele frequency were observed in the Gironde population compared to the Ster population, this trend being however not significant ($p>0.05$). A moderate decrease in the CK A allele frequency was observed in the Gironde estuary with respect to the reference site (Ster). The p53 A allele frequency was globally higher ($p>0.05$) in contaminated populations (Gironde, Loire, Seine and Vilaine) compared to the "reference" site (Ster); this increase ranging from 12.4% (Gironde and Loire) to 13.7% (Seine). In the Vilaine estuary, a mean increase of 10% in BHMT and PGDS A allele frequencies was observed compared to the Ster estuary whereas a 12.5% decrease in PGDS A allele frequency was observed for Seine compared to Ster. A 10% decrease in PGDS B allele frequency was also observed in Vilaine and Gironde estuaries compared to the reference site (Ster).

Mean observed heterozygosities were globally higher in the Ster and Gironde populations (Ho=0.36 and 0.39 respectively) than in the Loire, Seine and Vilaine populations (Ho=0.32, 0.34, 0.29 respectively), but not significantly different (all t-values < 1.48, $p>0.05$, with 4 df). A significant heterozygote deficit ($Fis>0$, $p<0.01$) was detected for GAPDH locus in all estuaries (Table 6). Globally, no significant departure from Hardy-Weinberg equilibrium was detected for the other loci. However a moderate heterozygote deficit for p53 locus was observed for the Seine and Loire populations ($p>0.05$), whereas a moderate heterozygote excess was observed in the Vilaine and Gironde populations ($p>0.05$) (Table 6). Moreover, a marked but un-significant heterozygote deficit was observed for the BHMT locus in the Loire estuary. No departure from Hardy-Weinberg equilibrium was observed for the PGDS locus (Table 6).

Genetic differentiation between populations

On the whole data set (Table 7), multi-locus Fst estimation indicated a moderate level of genetic differentiation (Fst = 0.005, $p>0.05$) between populations, confirmed by a non-significant exact test of genic differentiation ($p=0.07$). Single locus Fst and exact tests suggested that particular loci (CK, p53 and principally PGDS) could explain a possible differentiation between populations (Table 7).

The estimation of Fst by locus and by pair of estuaries indicated that several pairs of populations displayed genetic differentiation (Table 8). For GAPDH, a genetic differentiation was observed between the Gironde and Loire populations as well as between the Gironde and Seine populations ($p<0.05$). For CK, a differentiation was detected between the Seine and Gironde populations ($p<0.05$). For p53, a general differentiation was found between the Ster (reference site) and the four contaminated populations (Table 8), but this trend was however not significant ($p>0.05$). For BHMT, the genetic differentiation was better marked between Ster and Vilaine populations, than between Gironde / Vilaine or Seine / Vilaine (Table 8). For PGDS locus, a significant differentiation was observed between Seine and Vilaine and between Seine and Gironde ($p<0.05$) (Table 8). However, after performing a FDR test on the levels of differentiation detected by locus and by pair of populations, the differentiations for the PGDS locus remained significant.

Allele sequencing

The sequences of the different alleles revealed both exonic and intronic polymorphism (Figure 3). Mutations detected for GAPDH, CK, BHMT and p53 were all identified as 1) synonymous (silent) mutations (no amino acid replacement) and/or 2) mutations in the non-coding regions (introns). On the contrary, 3 out of the 5 alleles detected in the PGDS locus (B, C and E alleles) displayed a fragment with a polymorphism resulting in a modification of the corresponding amino acid, the sequence of allele A being used as a reference: valine was changed by alanine for allele B, aspartic acid by glutamic acid for allele C, and valine by isoleucine for allele E (Figure 3). The SWISS Model 3.5 was used to determine the secondary structure of the different PGDS variants but revealed no difference.

4. Discussion

Molecular characterization

The exon/intron structures obtained for the different genes in this study were compared to the very few known gene structures available in the databases (essentially mammal's sequences). Sequences of the creatine kinase gene (MM-CK gene) from *Platichthys stellatus* (**GU324256**) and *Homo sapiens* (**NC_000019**) show the same structure than the sequence identified for *P. flesus*, with 7 exons (same length) and 6 introns, the length of the introns being however slightly different for *P. stellatus* and largely different for *H. sapiens* compared to *P. flesus* sequence. Indeed, the DNA sequence length of *H. sapiens* reaches 16463 bp compared to 2369 bp and 3091 bp for respectively *P. flesus* and *P. stellatus*. The comparison of the GAPDH gene between *P. flesus* and *H. sapiens* (**NG_007073**) shows the same structure with 11 short exons (however not of the same length). The coding sequence length differs between the two species (999 and 1226 bp respectively for *P. flesus* and *H. sapiens*) a well as the length of the gene (4086 and 11908 bp respectively). The BHMT and PGDS genes also show the same structure between *P. flesus* and *H. sapiens* (**NC_000005** and **NC_000009** for respectively BHMT and PGDS), the length of the genes being however largely different: 2534 bp and 20510 bp for the BHMT gene and 1465 bp and 4239 bp for the PGDS gene for *P. flesus* and *H. sapiens* respectively.

Genetic variability within populations

Flounder populations showed limited departure from Hardy-Weinberg equilibrium, except for the GAPDH locus, where strong heterozygote deficits were observed. These deficiencies for GAPDH locus may be explained by technical artefacts such as the existence of null allele and/or the poor allele discrimination (Lundy et al., 1999); these deficiencies being observed in all five studied populations.

Other loci displayed limited values of heterozygote excess or deficit, although not at a significant level. The CK locus appeared to be at Hardy-Weinberg equilibrium, as Fis values were very close to zero. For the three other loci (p53, BHMT, PGDS), only some populations showed heterozygote excess or deficit. This situation is not indicative of a Wahlund effect, since populations that deviate from Hardy–Weinberg expectations are different according to the loci. Non random reproduction and small size of the natural populations are also unlikely, because flounder population sizes are generally considered as important in large estuaries of the French coast (Masson, 1988). Strong genetic mixing occurs generally during the reproduction period by the gathering of individuals for spawning (Berrebi, 1988; Masson, 1988; Borsa et al., 1997), thus non random reproduction is unlikely. A sampling bias could be at the origin of these differences, but the existence of a selective pressure acting on these three

loci (p53, BHMT, PGDS) in contaminated areas could be also suggested, as it was in previous studies focusing on the same fish species and in the same estuaries (Laroche et al., 2002; Marchand et al., 2004). In these last studies, several allozyme loci were probably submitted to a selective pressure induced by the chemical stress.

Genetic differentiation

We hypothesised the presence of null alleles for GAPDH locus, thus the genetic differentiation linked to this loci is probably biased. Consequently, the previous loci will not be individually considered in the following discussion on the genetic differentiation.

Global multi-locus estimation of Fst between populations indicated a low level of genetic differentiation, with PGDS explaining the majority of this differentiation. This result suggests a high gene flow between populations. Since the marine environment is generally considered as a highly dispersive environment, low levels of genetic structure are commonly observed for marine species (Ward et al., 1994; Graves, 1998; Waples, 1998). For the flounder, the possible gathering of adults stemming from different areas in spawning aggregations at the mouth of the estuaries, combined with the high dispersal potential of its pelagic eggs and larvae (several weeks in the plankton), may explain effective connection between populations (Borsa et al., 1997). The previous authors also found a weak pattern of differentiation by distance for flounder populations from the South-Western Baltic Sea to Southern Portugal with allozyme markers. However, the weak genetic structure detected in our study, may also be linked, as for allozymes, to a reduced genetic variability of our DNA markers showing a limited allelic diversity (between two and five).. Moreover, the genetic differentiation detected between the Gironde and the Seine estuaries, particularly at the CK and PGDS loci, may be more related to the geographical distance than to a differential pollution context, since these estuaries are the most distant over the whole data set.

However, considering neutral markers like microsatellites, flatfish populations are generally weakly structured over large geographical distances in the North East Atlantic; this trend was particularly observed for the flounder (Hemmer-Hansen et al., 2007a), the plaice (Hoarau et al., 2002) and the sole (Rolland et al., 2007). Thus, we suggest that the patterns of genetic structuration detected for the flounder in our study are probably more linked to local selection of contrasted environments on the candidate genes than to the consequence of geographical distance between estuaries (*i.e.* possible isolation by distance, or different history of colonization). A convergent hypothesis was formulated by Hemmer-Hansen et al. (2007b) working on the genetic structure of flounder populations in the northern part of the

species distribution, and considering a candidate gene (*Hsc 70*) and microsatellites; they concluded that the genetic structure among Atlantic and western Baltic Sea samples was more related to alternative factor (environmental gradient) than to the geographical distance itself.

Despite the low global multi-locus genetic differentiation, the single locus differentiation between pairs of populations and the distribution of allelic frequencies give a more subtle vision of the genetic structure. The genetic differentiation, considering a particular locus and the different pairs of populations could be linked to the potential selective pressure of the cocktails of contaminants, the analysis of pollutants in fish tissues displaying contrasted patterns of contamination (1) between the reference and the contaminated estuaries, and (2) between the contaminated estuaries (Evrard et al., 2010). One sequence polymorphism was identified at codon 134 of the p53 locus. It was identified previously as well as twenty one other polymorphisms in *P. flesus* from different locations (Cachot et al., 2000, Franklin et al., 2000). Pairwise Fst values for the p53 locus indicated that a genetic differentiation was observed between the Ster estuary (reference site) and the other contaminated estuaries (however non-significant). This difference might be related to the chemical stress and potentially reflects the existence of pollutants acting as selective agents in chronically contaminated populations in large estuaries. The distribution of allelic frequencies highlighted several convergences between contaminated sites compared to the reference site; thus an increase of the frequency of the A allele was observed in contaminated estuaries with respect to the Ster. As p53 is a crucial protein involved in cell growth control and maintenance of genome integrity, individuals carrying the p53 A allele could potentially be selected by chemical pressure. The hypothesis of a possible “resistant character” associated to the p53 A allele in contaminated systems is formulated. The selective pressure may select A alleles: (1) directly, through their differential efficiency (particular alleles being more efficient than others) or (2) indirectly, through hitchhiking phenomenon (linkage disequilibrium, e.g. non-random, interdependent, arrangement of alleles at different loci). The polymorphism observed at exon 4 conducts to silent mutations, privileging thus the hypothesis of a hitchhiking phenomenon acting on this locus. Nevertheless, such hypothesis should be supported by larger sample sizes and temporal replicates to strengthen the results. Genotype-phenotype coupling as well as gene expression on p53 should also be performed in the future, to explore the functional role of the p53 polymorphism.

For the PGDS locus, a significant genetic differentiation between estuaries was found. Moreover, an increase of the A allele at this locus is observed in the Vilaine estuary compared

to the Ster and to other estuaries. PGDS is an enzyme involved in important metabolic processes i.e. the synthesis of prostaglandins (Inoue, 2008) and some authors underlined that it may be involved in xenobiotics detoxification (Vogel, 2000). Given that the Vilaine estuary is submitted to a pesticide stress due to intensive agricultural practises and because PGDS is differentially expressed in response to pesticides exposure (Marchand et al., 2006), it is hypothesized that the cocktail of pesticides of this estuary acts as a selective agent on the PGDS locus, favouring the A allele. Mutations detected for PGDS lead to amino acid changes but however no difference in the secondary structure of the different variants was detected; indeed, valine, alanine and isoleucine (alleles A, B and E) belong to non-polar hydrophobic amino acids and acid aspartic and acid glutamic (allele C) are both acidic polar amino acids. Selective pressure potentially detected on this locus may thus act indirectly on the different protein sequences through hitchhiking phenomenon as it is hypothesized for p53.

Perspectives

New investigations are developed now in our laboratory, on the previous flounder populations stemming from environmentally contrasted estuaries; the main objective is to confirm the possible selective effects of the contaminants on the candidate genes investigated in this study: (1) by increasing the sample sizes of each population as well as examining temporal replicates to strengthen the results found in this study, (2) by comparing the genetic structure of the populations found with the previous candidate genes *versus* the structure found with neutral markers like microsatellites; neutral loci across the genome will be similarly affected by demography and the evolutionary history of populations, and loci under selection will behave differently and therefore reveal 'outlier' patterns of variation (Luikart et al., 2003); and (3) by exploring thoroughly the possible couplings between genotypes and phenotypes (associated gene expression, load of contaminants, DNA damages, liver pathologies) in the field.

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Table 1: Primers designed for cDNA amplifications. The F and R letters found in the Primer name indicate respectively forward (F) and reverse (R) primers; Ta: annealing temperatures of the different primers.

Primer name	Primer sequence	Ta (°C)
GAPDH F_1	5'-GTGTCTTCACCACCATTGAGAAGGC-3'	61
GAPDH R_1	5'-ATTGGCTGCTCGGTTTACTCCTTGGC-3'	65
CK F_1	5'-GGTTCTGACCAAGGAGCTGTATGGC-3'	62
CK R_1	5'-GGACACGGCTGGACAGAACATAGTTG-3'	62
PGDS F_1	5'-ACGTTGTCGGCTATGCCTCCAACG-3'	65
PGDS R_1	5'-CGGCTGAGGAGGTTGTTGAGGATG-3'	64
BHMT F_1	5'- ACACTGGGAAACAGAGATGGCACCT-3'	62
BHMT R_1	5'-TCCAGTGATACTGAGTGTCTGACCC-3'	57
Oligo dt Race primer	5'-GACCACGCGTATCGATGTCGAC$T_{(16)}$-3'	72
Race primer	5'-GACCACGCGTATCGATGTCGACT-3'	61

Table 2: Primers designed for gene amplifications (F: forward, R: reverse and Ta: annealing temperature).

Primer name	Primer sequence	Ta (°C)	Fragment amplified
GAPDH F_2	5'-ATGGTGAAAGTTGGTATCAATGG-3'	55	1993 bp
GAPDH R_2	5'-CTCAATGGTGGTGAAGACACCGGTGG-3'	65	
GAPDH F_3	5'-GTGTCTTCACCACCATTGAGAAGGCC-3'	63	677 bp
GAPDH R_3	5'-TGGGATGATGTTCTGGCTGGCACCGC-3'	66	
GAPDH F_4	5'-AACATCATCCCAGCTTCAACTGGTGCCGC-3'	66	1060 bp
GAPDH R_4	5'-ATTGGCTGCTCGGTTTACTCCTTGGC-3'	63	
CK F_2	5'-ATGCCTTTCGGAAACACCCACAACA-3'	59	1963 bp
CK R_2	5'-AGATGAGATTGTTCCTCTCTACTT-3'	55	
PGDS F_2	5'-AGCAACATGATGAACTCGCT-3'	52	1123 bp
PGDS R_2	5'-TCAGGCCCGGTGACATTGGA-3'	58	
BHMT F_2	5'-CACTGGGAAACAGAGATGGCA-3'	57	1085 bp
BHMT R_2	5'-AAGATGCCCTTCACTTCTGTCTC -3'	57	
BHMT F_3	5'- GAGACAGAAGTGAAGGGCATCTT -3'	57	938 bp
BHMT R_3	5'- GTGTGCATCTCCAGACCAGCG-3'	60	
BHMT F_4	5'- AGAGAGGCCTACAAGGCTGGA-3'	59	487 bp
BHMT R_4	5'- TTGACCGAGGACACTCTCGC -3'	58	

Table 3: Primers designed for PCR-SSCP amplifications (F: Forward, R: Reverse and Ta: annealing temperature).

Primer name	Primer sequence	Ta (°C)	Fragment amplified
GAPDH F_5	5'-CCTTGTCTCCTCCCTCTTTAG-3'	56	380 bp
GAPDH R_5	5'-GTTGAGGACTGAGTACGTAC-3'		
CK F_3	5'-TCCTCTTTCTTATTTAACAG-3'	55	364 bp
CK R_3	5'-AGATGAGATTGTTCCTCTCTACTT-3'		
PGDS F_3	5'-TGTGTATTCCTGCTTTATCAG-3'	58	371 bp
PGDS R_3	5'-GCATTGATTCAAAAGCGTCTG-3'		
BHMT F_4	5'-TGTGTTATTTTCAATAGTGCG-3'	58	151 bp
BHMT R_4	5'-ATACAGTGGTTGAACACTTAC-3'		
p53 F	5’-GGCTTTCTCCCCGTGTTTCTCTCAG-3’	55	221 bp
p53 R	5’-TACACTGATCACTTTACTTAC-3’		

Table 4: Molecular characterization

Gene name	GAPDH	CK	PGDS	BHMT
cDNA accession number	**AJ937522**	**FN432387**	**FN432388**	**FN432389**
DNA accession number	**AJ937521**	**FN432390**	**FN432391**	**FN432392**
Coding region length (bp)	999	1146	540	1206
Number of amino acids	332	381	179	401
Molecular weight (kDa)	35.87	43	20.15	43.98
Isoelectric point	8.27	6.22	5.86	7.22
5’UTR length (bp)	73	93	25	67
3’UTR length (bp)	329	331	343	176
DNA length (bp)	4086	2369	1465	2534
Number of introns	10	6	5	7
Number of exons	11	7	6	8

Table 5: Allelic frequencies for each location and each locus. N = number of fish per estuary; Ho: mean observed heterozygosity; He: mean expected heterozygosity.

		Ster	Gironde	Loire	Seine	Vilaine
N		40	40	39	40	30
Ho		0,360	0,390	0,323	0,340	0,287
He		0,376	0,411	0,386	0,393	0,315
Locus	**Allele**					
GAPDH	**A**	0,675	0,563	0,667	0,637	0,683
	B	0,163	0,300	0,128	0,138	0,167
	C	0,125	0,138	0,205	0,188	0,150
	D	0,013	0,000	0,000	0,025	0,000
	E	0,025	0,000	0,000	0,013	0,000
CK	**A**	0,887	0,813	0,872	0,925	0,917
	B	0,050	0,050	0,051	0,063	0,033
	C	0,063	0,087	0,038	0,000	0,033
	D	0,000	0,050	0,038	0,013	0,017
p53	**A**	0,388	0,512	0,513	0,525	0,517
	B	0,613	0,487	0,487	0,475	0,483
BHMT3	**A**	0,825	0,850	0,872	0,850	0,933
	B	0,175	0,150	0,128	0,150	0,067
PGDS	**A**	0,738	0,775	0,679	0,613	0,833
	B	0,263	0,163	0,295	0,338	0,150
	C	0,000	0,050	0,013	0,037	0,017
	D	0,000	0,013	0,013	0,000	0,000
	E	0,000	0,000	0,000	0,013	0,000

Table 6: Estimation of inbreeding coefficient (Fis) by locus for each location. Significance of Fis was assessed by Fisher's exact tests. Bold values indicated significant p-values, with ** for p<0.01 and *** for p<0.001.

	Ster	**Gironde**	**Loire**	**Vilaine**	**Seine**
GAPDH	**0.363*****	**0.358****	**0.494*****	**0.529*****	**0.591*****
CK	-0.080	-0.057	-0.081	-0.043	-0.056
P53	-0.093	-0.138	0.089	-0.252	0.110
BHMT	-0.026	0.032	0.323	-0.054	0.032
PGDS	-0.085	-0.067	-0.067	0.074	-0.215

Table 7: Single and multi-locus global assessment of genetic differentiation between estuaries using (1) Fst estimated with Weir & Cockerham's ∂ and tested using 5000 permutations (* p<0.05) and (2) exact test (p-values) of genetic differentiation tested with the Markov chain method, using 3000 iterations.

	Multilocus	**GAPDH**	**CK**	**p53**	**BHMT**	**PGDS**
θ	0.005	-0.001	0.005	0.002	-0.003	0.021*
Exact test	0.07	0.184	0.213	0.372	0.415	**0.031**

Table 8: Estimation of single-locus pairwise θ values with associated significance in bold. * for p<0.05.

θ	**GAPDH**	**P53**	**CK**	**BHMT**	**PGDS**
Ster – Seine	-0,01278	0,02507	0,00442	-0,01035	0,01445
Ster – Vilaine	-0,0198	0,0213	-0,00766	**0,03744***	0,01737
Ster - Gironde	0,01252	0,02025	0,00452	-0,01035	0,00676
Ster - Loire	-0,00986	0,01883	-0,0065	-0,00586	-0,00614
Seine – Vilaine	-0,01828	-0,01399	-0,00705	0,01928	**0,08133 ***
Seine – Gironde	**0,01258 ***	-0,01216	**0,0333 ***	-0,01307	**0,05131 ***
Seine – Loire	-0,01796	-0,01382	0,00177	-0,01298	-0,00337
Vilaine – Gironde	0,00891	-0,01196	0,01537	0,01928	-0,00726
Vilaine - Loire	-0,01778	-0,01404	-0,00682	0,00276	0,04219
Gironde - Loire	**0,02263 ***	-0,01249	-0,00118	-0,01298	0,02149

Figure captions

Figure 1 Sampling sites: contaminated estuaries (Seine, Vilaine, Loire, Gironde) and reference estuary (Ster)

Figure 2: Molecular structure (exons/introns) of the 5 genes characterized: A) GAPDH, B) CK, C) BHMT, and D) PGDS. The length of each exon and intron is indicated below (bp). Fragments amplified by SSCP are also indicated.

Figure 3: Sequences of the different alleles detected for the 5 locus studied.


ENGLISH CHANNEL
Seine
Ster
Vilaine
Loire
BAY OF BISCAY
Gironde
km
0
50
100
N

Figure 1

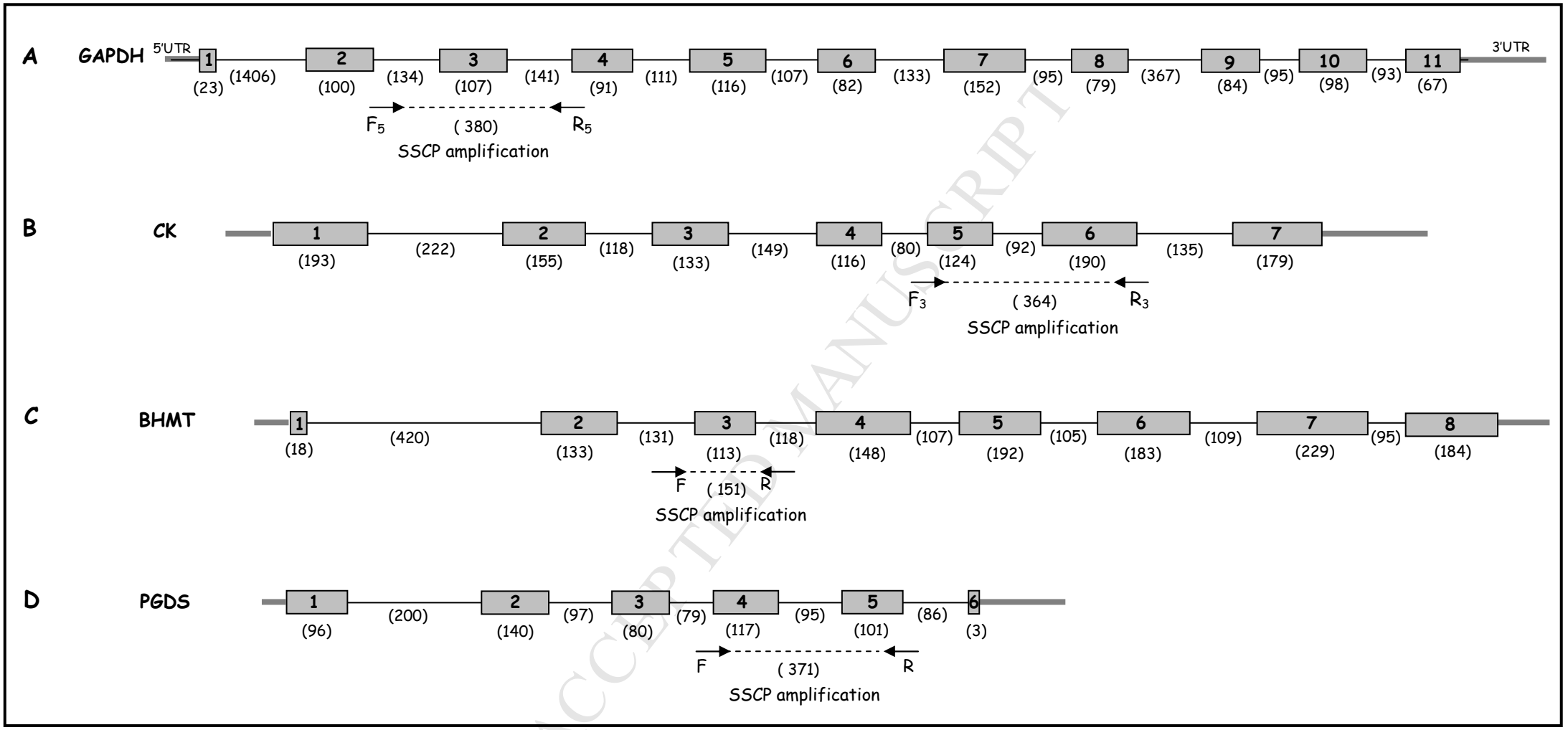

Figure 2